Targeting Myeloid Derived Suppressor Cells (MDSC) Using a Novel Adenosine Monophosphate-activated Protein Kinase (AMPK) Activator: OSU-53.

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I. Introduction

A. Tumor Microenvironment

The tumor microenvironment (TME) is composed of various cell types that includes fibroblasts, endothelial cells, tumor cells, and immune cells (Figure 1). The presence of inflammatory cells in the tumor stroma has been shown to facilitate tumor development, both in experimental models and in the pathogenesis of human cancers. Understanding this TME and immune cell recruitment is vital in the pursuit of tumor degradation. It has been shown that cancer cells in the TME secrete a variety of cytokines and chemokines that actively recruit immune cells into the TME by establishing autocrine and paracrine signaling pathways, which directly and indirectly suppress the immune system (Bissell and Hines 2011). Once recruited, the immune cells promote tumor growth through two opposing mechanisms. On one hand, TAMs secrete cytokines and proteases that facilitate tumor growth and angiogenesis. In contrast, MDSC promote tumor growth by suppressing the function of normal immune cells, which are responsible for the destruction of tumor cells.

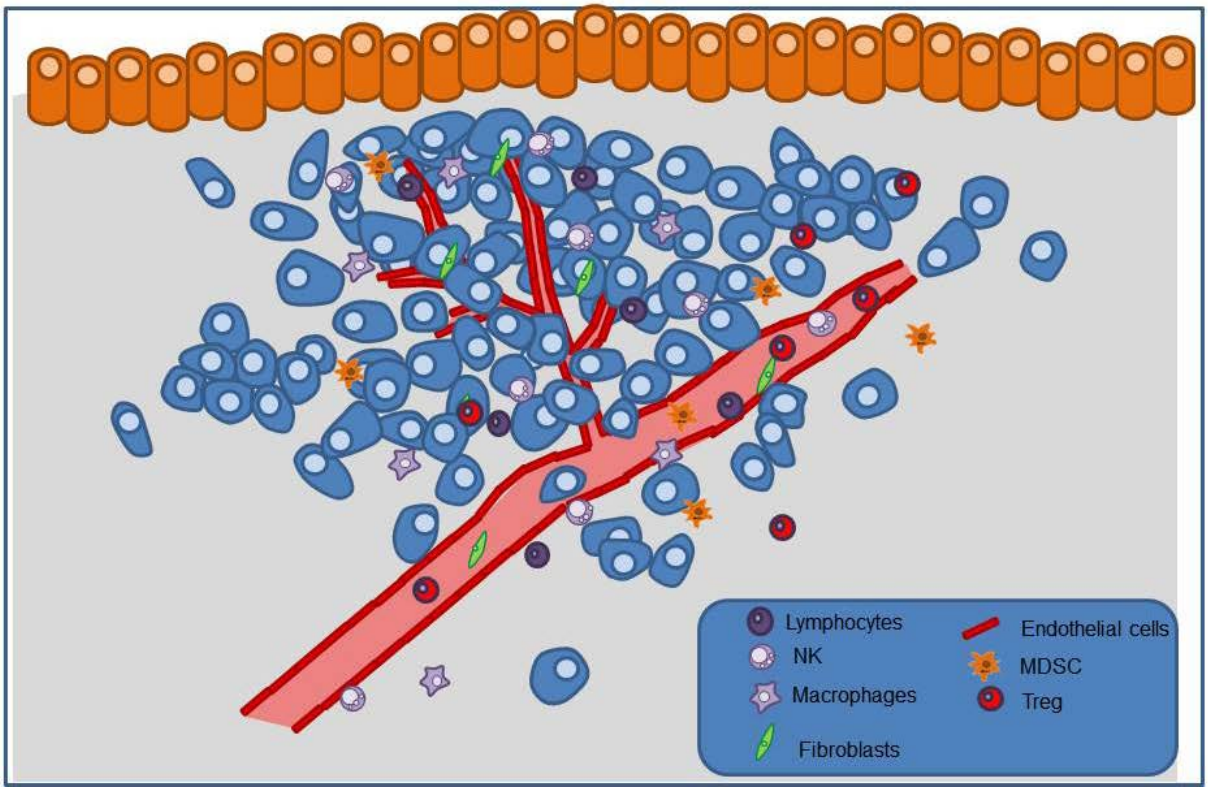


Figure 1: MDSC are constituents of the tumor microenvironment.

B. Myeloid Derived Suppressor Cells

MDSC represent a heterogeneous population of myeloid cells composed of macrophages, granulocytes and dendritic cells that are at early stages of development. Under physiological conditions they represent up to 20-30% of bone marrow cells and ~4% of nucleated cells in the spleen (Youn et al. 2008). However, under pathological conditions such as infections, stress, and cancer there is an accumulation of MDSC in lymphoid organs (Trikha and Carson 2014).

The presence of MDSC was first reported in the blood of patients with head and neck cancer, and they were identified as $Lin^{-}CD33^{+}CD15^{+}CD34^{+}$ cells (Almand et al. 2001). Human MDSC are now broadly classified as $CD33^{+}HLADR^{low/neg}$. In mice, MDSC are identified by expression of the myeloid markers Gr-1 and CD11b. Human and murine MDSC are divided into two categories: graulocytic and monocytic. Murine models are represented as G-MDSC and M-

MDSC while human monocytic MDSC express CD14 and granulocytic MDSC express CD15 (Mandruzzato et al. 2009; Vuk-Pavlović et al. 2010).

MDSC can suppress the function of normal immune cells, such as NK cells and T cells, by creating a suppressive environment in the tumor stroma (Trikha and Carson 2014). Studies have shown that T cell function is suppressed by MDSC by a variety of mechanisms such as the release of immune-suppressive cytokines, arginase, nitric oxide and reactive oxygen species (ROS) (Gabrilovich and Nagaraj 2009; Bissell and Hines 2011). The release of these factors leads to decreased immune cell function and helps facilitate tumor progression.

C. Signaling Pathways

A number of signaling pathways are implicated during MDSC development and in the regulation of their function (Condamine and Gabrilovich 2011). It is widely believed that perturbation of signaling pathways involved during myeloid development under pathological conditions such as cancer can contribute to the development of MDSC. A number of pathways are thought to be linked to MDSC function: Ras, PI3K, IL-6, JAK/STAT, TGF-ß, PGE2 and Cox2 (as shown in Figure 2 below).

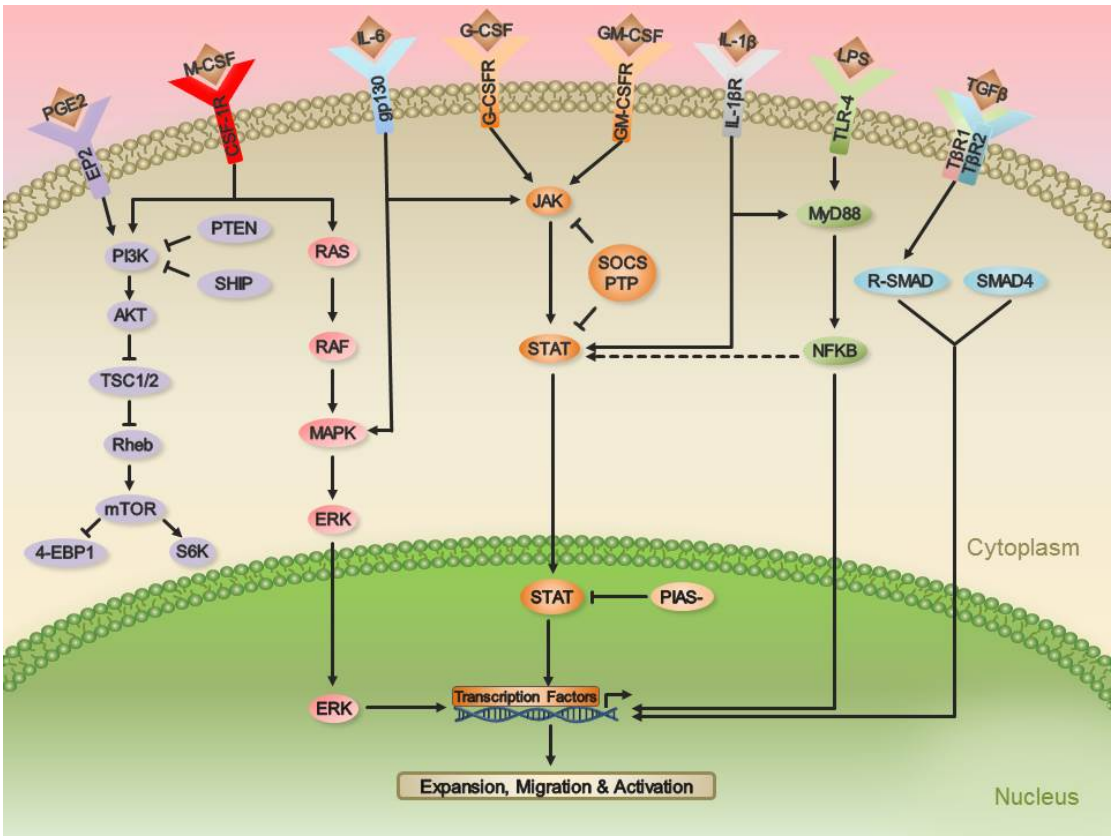


Figure 2: Signaling pathways that affect the proliferation and maturation of cancer cells

Inflammation plays a key role in alerting the body of an intruder during tumor progression and general immune defense. IL-6 is a pro-inflammatory cytokine that regulates many downstream pathways such as signal transducer and activator of transcription 3 (STAT3). This regulation directly leads to cancer cell proliferation, survival, and migration. Additionally, it has been shown that up-regulation of IL-6 leads to an increased signaling in the Janus kinase 2 (JAK) / STAT pathway which has been linked to an increase in accumulation of MDSC in the TME (Mundy-Bosse et al. 2011). The JAK/STAT pathway plays a crucial role in mediating inflammatory response. Specifically, IL-6 is known to regulate the function and expansion of MDSC via JAK and STAT3 signaling pathway (Mace et al. 2013).

Another pathway affected by MDSC is the PI3K is the signaling pathway. This pathway catalyzes phosphorylation of the 3'OH on phosphatidylinositol in the plasma membrane (Trikha

and Carson 2014). PI3K signaling has been found to have effects on cell growth, survival, migration, and cellular metabolism. Following the PI3K pathway, the mTOR pathway has been shown to work in accordance with the adenosine monophosphate-activated protein kinase (AMPK) phosphorylation which is up-regulated during MDSC maturation. Therefore, AMPK inhibition can decrease the immunosuppressive potential of MDSC. We hypothesized that therapeutic agents like OSU-53 (an AMP kinase inhibitor) can target these signaling pathways, and should help in eliminating MDSC from the TME, while making immune-based therapies more effective.

II. OSU-53

A. Background

Adenosine monophosphate-activated protein kinase (AMPK) is an enzyme which plays an integral role in cellular energy homeostasis and cellular metabolism. When ATP storages begins to deplete during physical activity, AMPK is activated leading to energy conversion and metabolism through a series of pathways and networks. Moreover, recent studies have linked AMPK to cancer cell growth and survival (Inoki et al. 2003). AMPK can also down regulate the mammalian target of rapamycin (mTOR) pathway resulting in decreased tumor growth (Lee et al. 2011). Recently, an AMPK activator known as metformin has been reported to reduce risk and/or mortality in certain types of cancers (Li et al. 2009; Wright and Stanford 2009; Bodmer et al. 2010).

This finding led to the synthesis of OSU-53 (Figure 3), a PPAR γ-inactive derivative which directly stimulates AMP kinase by the Chen Laboratory (Lee et al. 2011). This compound targets tumor metabolism by inhibiting tumorigenesis via oncogenic pathway regulation (Lee et al. 2011).

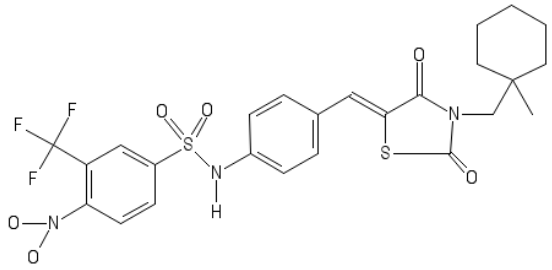


Figure 3: Chemical structure of the compound OSU-53

Additionally, AMPK activation has been shown to suppress the expression of the inflammatory cytokine IL-6, which plays a key role in promoting breast cancer progression via JAK2/STAT3 signaling (Berishaj et al. 2007). This led us to investigate the potential use of OSU-53, as therapeutic agent to eliminate or inhibit the suppressive activity of MDSC as a potential new therapy for cancer. This study was designed to investigate whether treatment of MDSC with OSU-53, could affect MDSC function by the utilization of a murine MDSC cell line known as MSC-2.

III. Materials and Methods

A. Cell Lines and Cell Culture

A murine breast cancer MDSC cell line, MSC-2 (obtained from (Apolloni et al. 2000)) was used for *in vitro* testing. Cells were cultured in RPMI medium (Life Technologies, Paisley, UK) with 10% FBS, 1% Anti-Anti, and 1% sodium pyruvate.

B. Annexin V-PI Flow Cytometry

Annexin- PI staining was used to determine cytotoxicity of the drugs on the MSC-2 cell line. The MSC-2 cells were treated with various doses of DMSO (vehicle) or OSU-53 (0-10 μM), and placed in an incubator at 37℃ in 5% CO_2 Cells were then harvested, re-suspended in annexin buffer, and stained with Annexin V and propidium iodine (PI). The cells were run on an LSRII flow cytometer (Becton-Dickinson, San José, CA) and then analyzed via Flowjo™ software (Treestar, Ashland, OR).

C. Western Blot

MSC-2 cells were cultured with varying amounts of OSU-53 for 18 hours. Cells were lysed via RIPA buffer containing protease and phosphatase (Sigma Aldrich, St. Louis, MO). The protein lysates were probed for AMPK, p-AMPK, and β–Actin antibodies.

D. Cell Migration Assay

The migration of MSC-2 cells treated with varying amounts of OSU-53 were measured in response to serum-free supernatant from a breast cancer cell line that has been engineered to over-express human HER2 (EMT6-HER2). Briefly, 5 x 10^4 transfected cells in serum-free medium were plated on an 8 μm transwell membrane. The lower compartment of the invasion chambers contained a conditioned supernatant from EMT6-HER2 cell cultures utilized as a chemoattractant. After overnight incubation at 37℃ the cells remaining on the upper surface of the membrane were remove, while the cells that migrated through the membrane were fixed, stained, and counterstained with Dip Quick Stain Kit (Jorgensen Laboratories, Inc., Loveland, CO). Photographs were taken of five 20X fields for each filter on an EVOS XL digital inverted microscope and cell numbers were enumerated using Image J software from the images (2048 x 1536 pixels) (Advanced Microscopy Group, Bothell, WA).

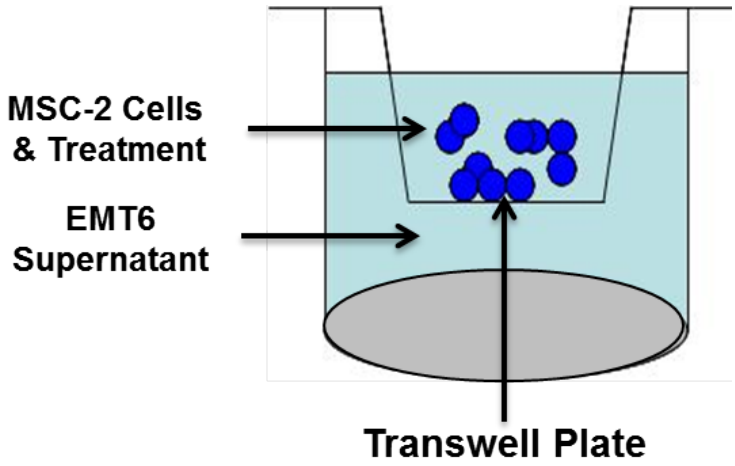


Figure 4: Boyden chamber model for cell migration assay

E. Nitric Oxide (NO) Estimation

MSC-2 cells treated with varying amounts of drug were cultured for 24 hours. After this period, the supernatant was harvested and quantified for NO using Griess Reagent (Sigma Aldrich, St. Louis, MO). Equal parts Greiss Reagent and sample were mixed together in a 96 well plate and absorbance values were recorded at 450 nm using a plate reader.

F. Enzyme-linked Immuno Assay (ELISA)

MSC-2 cells treated with varying amounts of drug were cultured for 24 hr. After this period the supernatant analyzed for presence of TNF-α and IL-6.via ELISA using a BIOPLEX kit.

G. Generation of MDSC

Normal peripheral blood mononuclear cells (PBMC) were obtained from normal healthy adult blood donors (source leukocytes, American Red Cross, Columbus, OH). PBMC were separated from source leujocytes by density gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences, Pittsburgh, PA) as previously described (Varker et al. 2006). PBMC were cultured in

media containing IL-6 and GM-CSF (Peprotech, Rocky Hill, NJ) for a period of six days at 37℃ in 5% CO_2. During this process, the PBMC were treated with a dose of 5 μM OSU-53 or DMSO vehicle control. The media and drug were changed every 2-3 days.

H. Statistical Analysis

The data was analyzed using a student's T test (Microsoft Excel).

IV. Results

OSU-53 treatment affects apoptosis of a murine MDSC cell line (MSC-2)

MSC-2, a mouse MDSC cell line that has been previously shown to mimic MDSC functions, was used to examine the effects of the AMPK activator OSU-53 on the function of MDSC *in vitro*. We first wanted to determine a concentration of the OSU-53 that was not cytotoxic to the cells. The MSC-2 cells were treated overnight with various doses with DMSO (vehicle) or OSU-53 (0-10 μM) to determine the dose at which OSU-53 was cytotoxic to MDSC cells. Annexin-PI staining showed that OSU-53 did not induce any apoptosis up to a dose of 5 μM (Figure 5A-5B). However, there was a significant increase in the number of apoptotic cells at a dose of 10 μM. Based on results of these experiments a dose range of 0-5 μM was selected for all subsequent experiments.

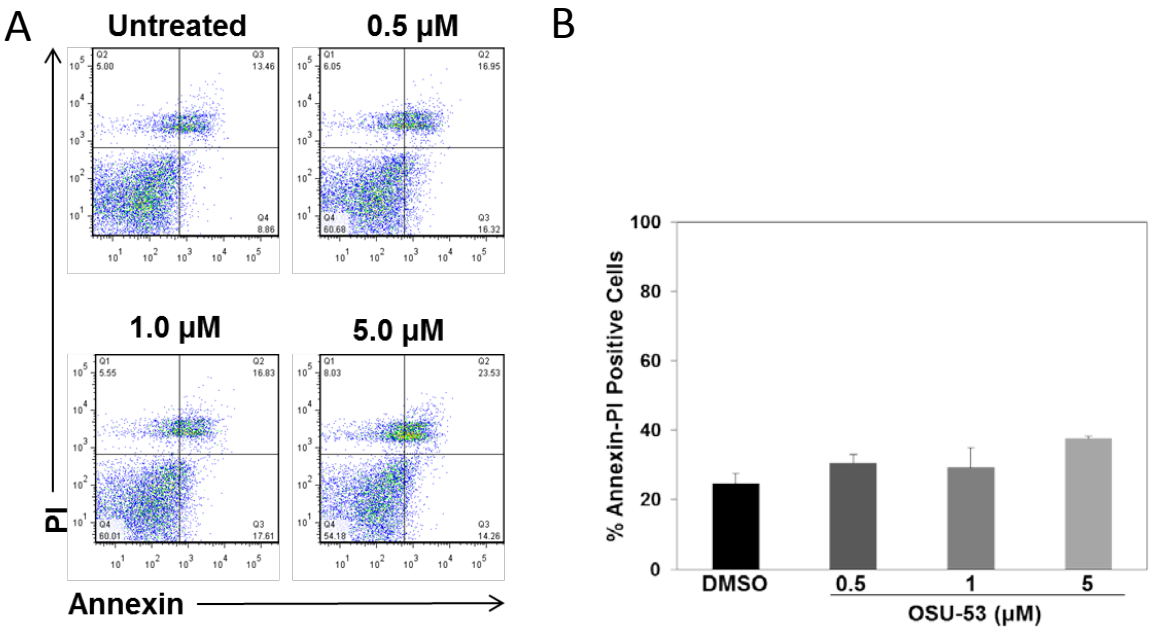


Figure 5: A&B. The effects of OSU-53 on apoptosis of a murine MDSC cell line. MSC-2 cells were treated with OSU-53 for 18 hours. Cells were then washed and stained with Annexin V-PI. **(A)** FACS profile of MSC-2 treated with OSU-53. **(B)** Percentage of apoptotic cells was measured by flow cytometry

OSU-53 treatment leads to the activation of AMPK in MDSC

OSU-53 is a PPAR-inactive derivative that has been shown to stimulate AMPK kinase in triple negative breast cancer cells (TNBC) (Lee et al. 2011) (Figure 6). We therefore wanted to investigate whether OSU-53 could also activate AMPK activity in MSC-2 cells. Western blot analysis showed that OSU-53 treatment did not affect the levels of AMPK in MSC-2 cells (Figure 6). However, there was an induction in the levels of phosphorylation of AMPK following the treatment of MSC-2 with 5 µM of OSU-53.

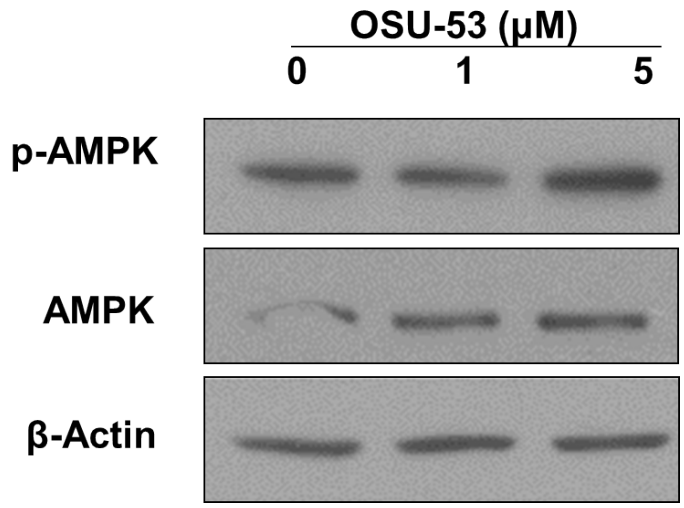


Figure 6: Effect of OSU-53 on phosphorylated AMPK. Western blot of MSC-2 cells treated with OSU-53 probed for p-AMPK, AMPK, and ß-Actin (loading control).

OSU-53 treatment leads to an attenuation of NO levels

Nitric oxide is one the key molecules produced by MDSC that is involved in immune suppression of NK and T cells functions (Bogdan, 2001). To examine the affect OSU-53 on the levels of NO, MSC-2 cells were treated with the indicated doses of OSU-53 and stimulated with LPS for 24 hours. Following the incubation, the supernatant were collected and analyzed for levels of nitrite using Griess reagent. Activation of MDSC with LPS resulted in an increase in the levels of nitrite to 25 μM. There was a 3-fold decrease in the levels of NO in the MSC-2 cells treated with 5 μM of OSU-53 (Figure 7).

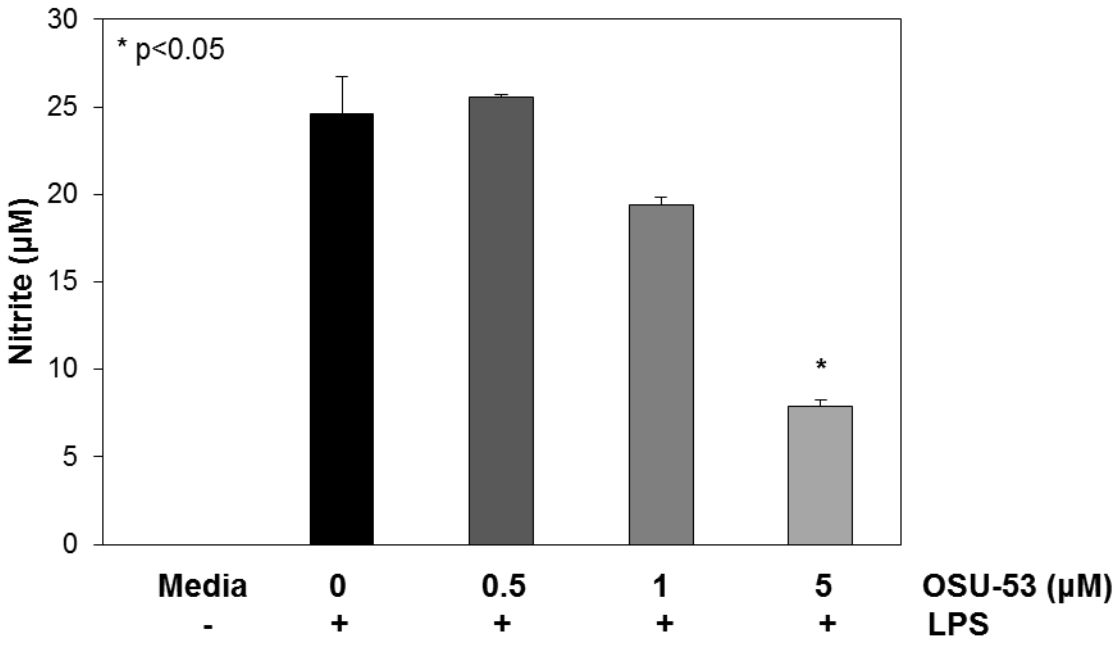


Figure 7: OSU-53 decreases nitric oxide production. MSC-2 cells were treated with indicated doses of OSU-53 for 12 hrs and then stimulated with LPS. The levels of nitrite were measured in the supernatant using Griess Reagent.

OSU-53 treatment reduces MDSC migration

Tumor cells secrete cytokines and chemokines that help in the recruitment of MDSC into the tumor microenvironment (Gabrilovich and Nagaraj 2009). We performed migration assay to determine whether treatment of MDSC with OSU-53 affected their ability to migrate in response to factors produced by tumor cells. These assays showed a significant reduction ($p<0.001$) in the number of MSC-2 cells treated with 5 μM of OSU -53 when compared to DMSO treated control (Figure 8).

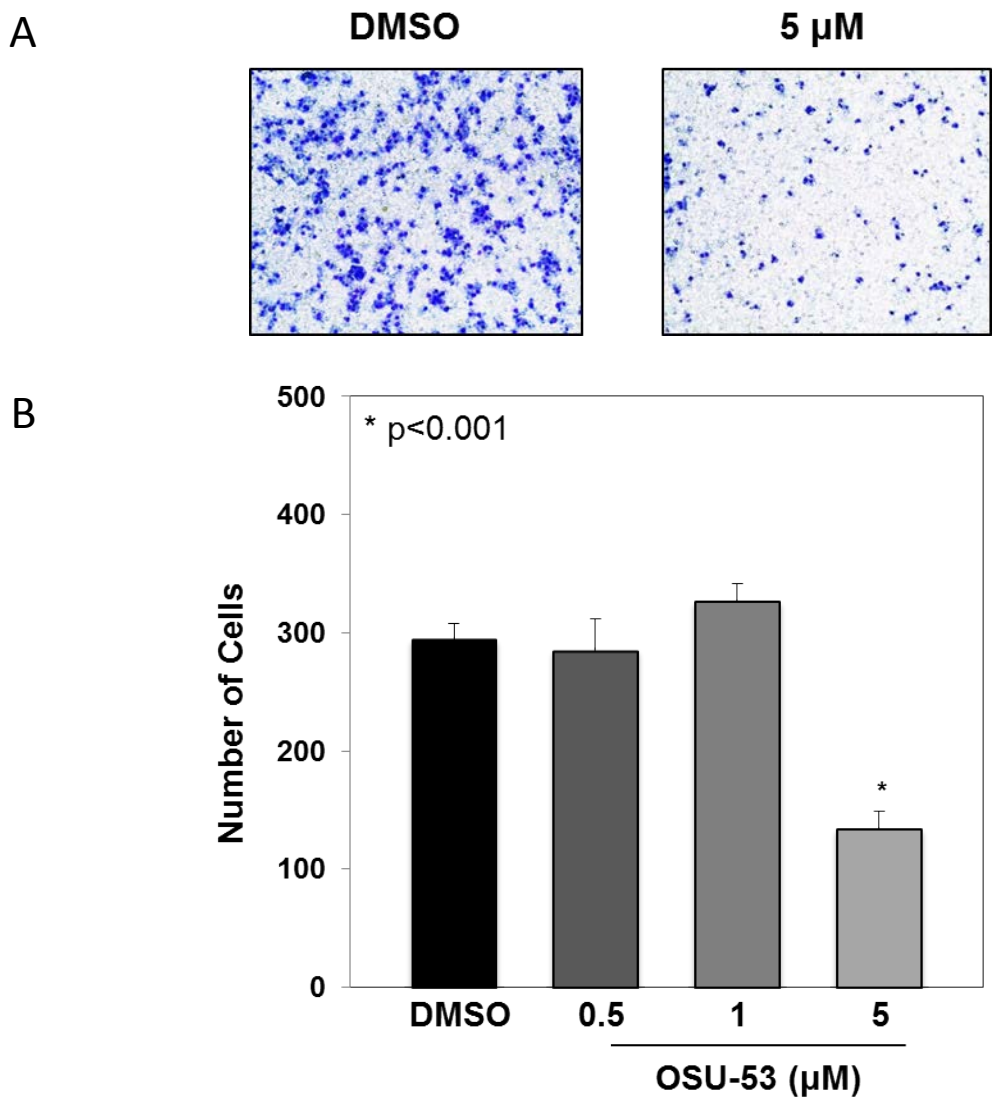
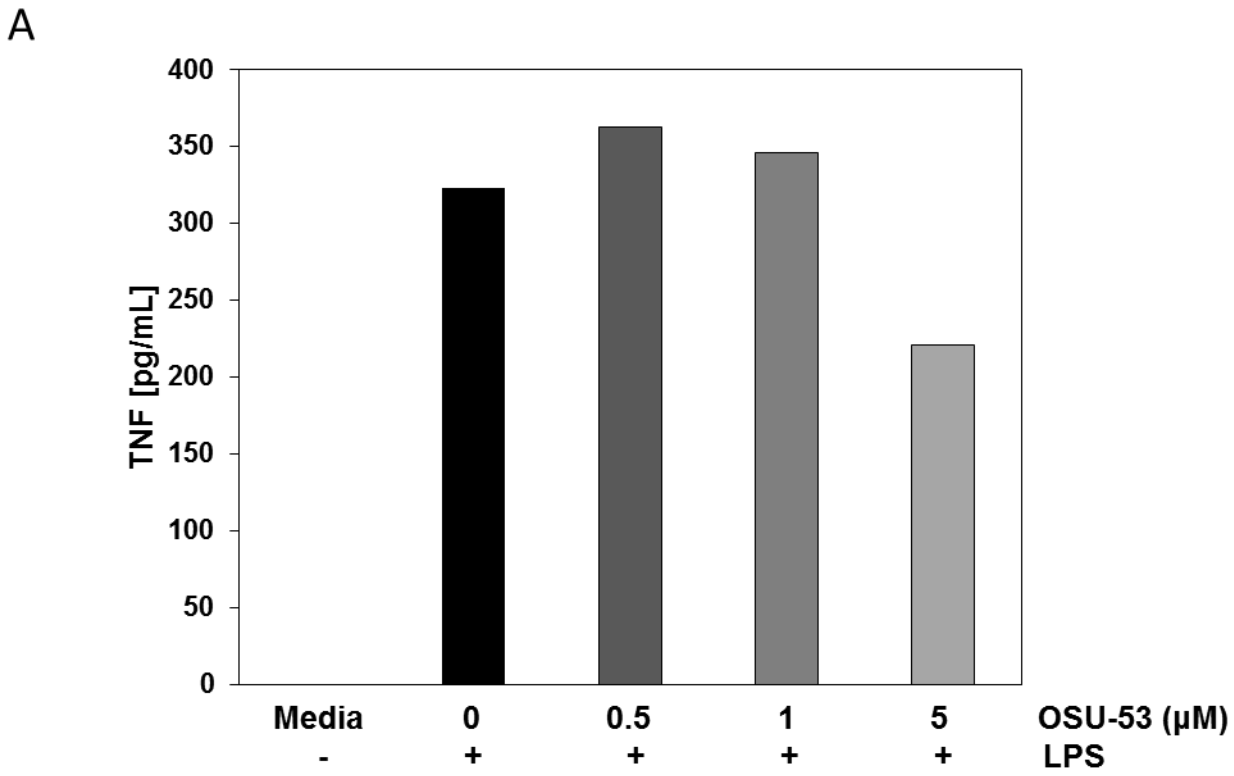


Figure 8: Effect of OSU-53 on cell migration. **(A)** Digital image of migrated MSC-2 cells. **(B)** OSU-53 treatment leads to reduced MDSC migration.

Reduced levels of IL-6 and TNF-α in OSU-53 treated cells

Since it has been shown that various signaling pathways are involved in the regulation of MDSC expansion and function (Trikha and Carson 2014), we next wanted to examine the whether OSU-53 treatment affected the secretion of pro-inflammatory cytokine like IL-6 and TNF-Aby MSC-2 cells. Data from the array of MSC-2 cells treated with varying doses of OSU-53 showed that MSC-2 cells activated with LPS did not produce detectable levels of IL-17, IL-10, IL-, IL-4,

INF-γ (data not shown). Interestingly, there was a significant increase in the levels of IL-6 and TNF-a by MSC-2 following stimulation with LPS. Moreover, treatment of MSC-2 cells with OSU-53 (5 μM) results in a reduction in the levels of IL-6 (2.9 fold) and TNF-α (1.5 fold) (Figure 9). Both these cytokine have been shown to have an important role in the regulation of MDSC function. Our results are consistent with the previous studies that have shown that OSU-53 can affect the levels of IL-6 in tumor cells. This is, however, the first report to our knowledge which shows that OSU-53 can modulate the levels of IL-6 in MDSC.



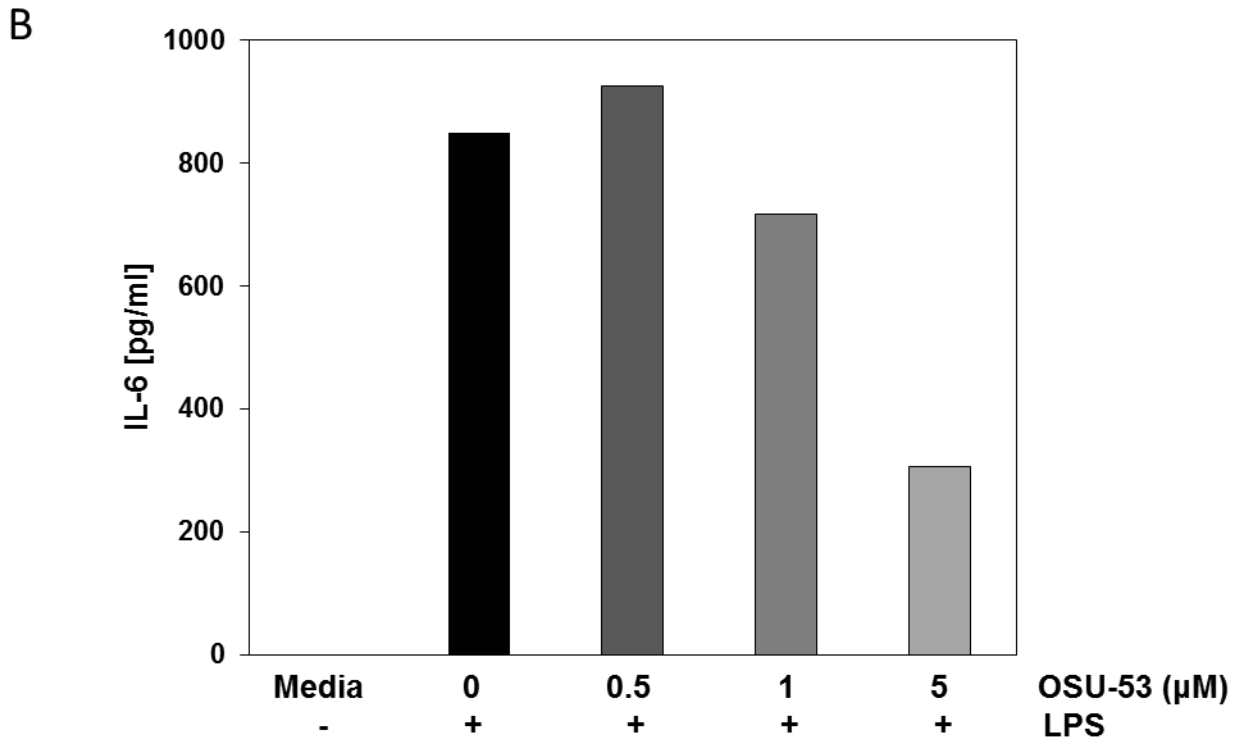


Figure 9: OSU-53 affects the level of cytokines measured by ELISA. **(A)** Relatively no effect on TNF-α. **(B)** OSU-53 decreases production of IL-6 cytokine.

MDSC generation is not affected by OSU 53

Cytokines like IL-6 and TNF-α are involved in the generation and expansion of MDSC during tumorigenesis (Condamine and Gabrilovich 2011). We therefore wanted to investigate whether treatment with OSU-53 could affect the generation of MDSC. PBMC treated with a DMSO control resulted in the generation of MDSC. The addition of OSU 53 did not impact the ability of PBMC to generate MDSC under these conditions *in vitro*.

V. Conclusion

- FACS analysis of cells treated with OSU-53 showed that 0.5-5 μM did not induce cell apoptosis.
- There was an increase in levels of activated p-AMPK after the treatment of MSC-2 cells with OSU-53.
- Treatment of MSC-2 with OSU-53 significantly ($p<0.05$) decreased NO production in MSC-2 cells by 1.5 fold.
- Cytokine levels of both IL-6 and TNF-α decreased in response to OSU-53 treatment.
- Cell migration was significantly ($p<0.05$) decreased in response to tumor supernatant after treatment of MSC-2 cells with OSU-53.

VI. Summary

Our results showed that OSU-53 was not cytotoxic at concentrations ranging from 0.5 μM -5 μM as determined by Annexin V staining. Interestingly, treatment of MSC-2 with OSU-53 (5 μM) led to a significant ($p<0.05$) reduction in the ability of these cells to migrate in response to tumor cells *in vitro*. There was also a decrease in production by MSC-2 cells following treatment with the drug. Furthermore, there was two-fold reduction in the levels of pro-inflammatory cytokine IL-6 which is linked to the activation of MDSC. In summary, OSU-53 can modulate MDSC function *in vitro* (Figure 10) by inhibiting the function of the murine MDSC cell line, MSC-2. Future studies will be aimed at investigating whether OSU-53 can modulate MDSC function in human models, and whether or not it can enhance antibody based therapy in tumor bearing mice.

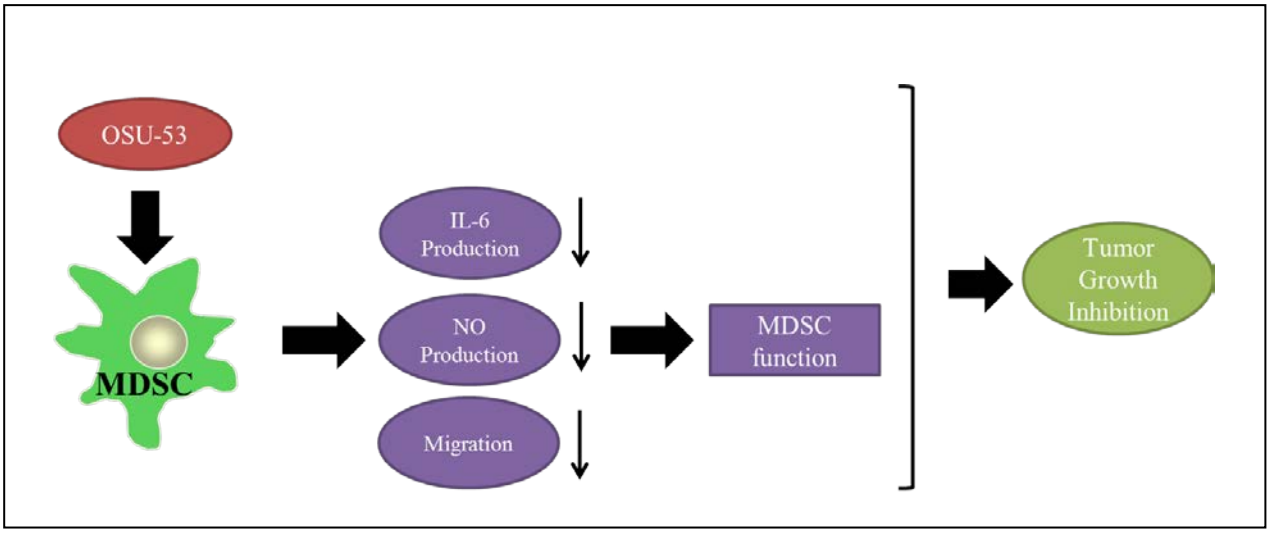


Figure 10: Effect of OSU-53 on MDSC function

VII. Acknowledgments

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